Reference: *Biol. Bull.*, **158**: 164–173. (February, 1980)

3,4-DIHYDROXYPHENYLALANINE (DOPA) AND SCLEROTIZATION OF PERIOSTRACUM IN *MYTILUS EDULIS* L.

J. H. WAITE[1] AND S. O. ANDERSEN

Zoophysiology Laboratory C, August Krogh Institute, 2100 Copenhagen, Ø. Denmark

The catecholic amino acid, 3,4-dihydroxyphenylalanine (DOPA), is present in specialized cells of most animals where it serves as an intermediate in the biosynthesis of catecholamine hormones, neurotransmitters and melanin pigments (Nagatsu, 1973).

A somewhat different role of DOPA is suggested by its occurrence in several invertebrate extracellular structural proteins including leech egg capsule (Knight and Hunt, 1974), trematode egg capsule (Rainsford, 1967), mytilid byssus fibers (Degens and Spencer, 1966) and the periostracum of various bivalve molluscs (Degens, Spencer and Parker, 1967; Waite, 1977; Waite and Andersen, 1978). All these structures share the property of being sclerotized by an undefined process of protein cross-linking termed quinone tanning (Brown, 1950; Brown, 1952). DOPA is the only known immediate precursor of quinones in these structures, but no experimental data exist about the role of DOPA in the protein cross-linking process.

In the periostracum of *Mytilus edulis,* DOPA is at all times an integral part of the structural protein and can not be dissociated from it without the complete hydrolysis of the protein. Waite and Anderson (1978) recently reported the recovery of significantly more DOPA from hydrolysates of new marginal periostracum than from older exposed periostracum. The apparent disappearance of DOPA might result from its modification by cross-linking to proteins, thus rendering the proteins less soluble with time, *i.e.*, sclerotization. To test this possibility, we have measured the amounts of DOPA and soluble protein in increasingly aged periostracal annuli in the shell of the mussel, *Mytilus edulis.*

Materials and Methods

Live mussels were collected from Issefjord (Denmark) during the months May to July. The age of each specimen was estimated by counting superficial annuli, and this estimate was corroborated by examination of umbonal cross-sections (Lutz, 1976). The periostracum was scrubbed clean under running tap water and peeled from the shells after partial decalcification in aqueous acetic acid (4%) and methanol (2.5%). Peeled periostracum was rinsed in three changes of distilled water and cut into 5 or 6 annular sections (Fig. 1). The ledge of periostracum along the shell margin is simply referred to as marginal periostracum. Each of the 39 sections was dehydrated in 95% ethanol, freeze-dried, pulverized and weighed. Powdered sections were subdivided into two lots—one to be assayed for soluble protein and, the other, for the determination of DOPA and tyrosine after acid hydrolysis. Soluble protein was extracted from periostracum with formic acid (13.0 M) at 4° C for 24 hr. Insoluble protein

[1] Present address: Department of Biochemistry, University of Connecticut Health Center, Farmington, Connecticut 06032.

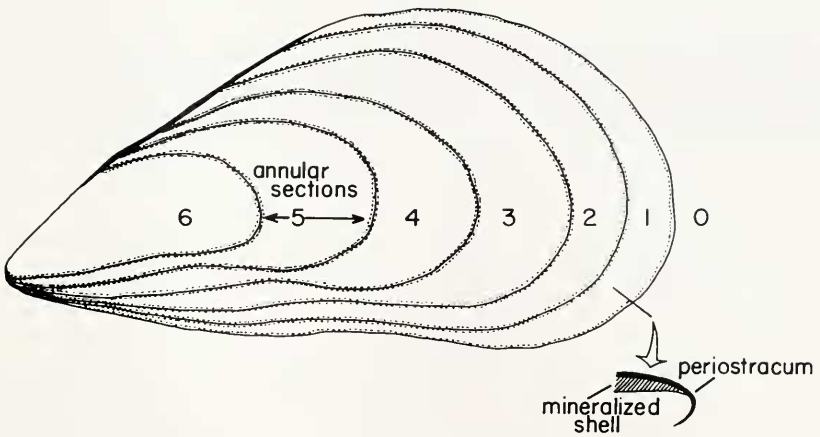

FIGURE 1. A drawing of the right valve of *Mytilus edulis* depicting the crescent-shaped annular sections into which the periostracum was cut. The insert (lower right) affords a cross-sectional view of the marginal periostracum. This is the only area where periostracum does not overlay mineralized shell. The numbers associated with the annular sections correspond roughly to the *average age* of a given section. Age is in years.

was removed by filtration through fritted glass; the filtrate was concentrated by rotary evaporation to a final volume of 2 to 4 ml. Aliquots of 2 to 4 μl or less were assayed for protein using a modified Lowry procedure (Hartree, 1972). Soluble protein from marginal periostracum was diluted 10-fold with distilled water, dialyzed against the same (12 hr at 4° C), freeze-dried and stored at −20° C.

Periostracal samples were hydrolyzed for 24 hr in 6 M HCl with 0.1% caprylic acid and 1.0% thiodiglycol at 110° C *in vacuo*. The acid was removed by vacuum evaporation at 50° C, and the residue was dissolved in 0.2 M acetic acid (2–3 ml) and centrifuged at 10,000 × *g* for 30 min to remove insoluble materials. Tyrosine and DOPA were separated as described previously (Waite and Andersen, 1978), and quantitated at 280 nm using the molar extinction coefficients 1180/(M·cm) [tyrosine] and 2600/(M·cm) [DOPA] (Merck Index, 8th Edition, 1968). Amino acid composition of three periostracal samples (soluble, marginal and first-annular periostracum) was determined after acid hydrolysis (described above) for 24, 48, and 72 hr on a Beckman Model 120 C analyzer according to the method of Moore (1968). Annuli older than one year were not analyzed for amino acids due to difficulty in insuring complete removal of attached extraneous marine flora and fauna.

Isolation of aromatic compounds other than DOPA and tyrosine from acid hydrolysates of periostracum (marginal) was performed according to the method of Waite and Andersen (1978) using gel filtration on Bio-Gel P-2 (50 × 1.5 cm) and ion exchange on cellulose phosphate in tandem. Eluants of each column were monitored for per cent transmittance at 280 nm. Isolated individual peaks were then scanned for absorbance between 225 and 325 nm with a Pye-Unicam SP-8000 spectrophotometer. Thin-layer chromatography on Merck cellulose Fertigplatten was used to check the purity of the isolated peaks. The solvent system was 4-parts 2-propanol to 1 part acetic acid to 1 part water, and the indicator was ninhydrin (0.1%) in absolute alcohol.

Results

An apparent increase in the age of periostracum is accompanied by decreasing solubility of periostracal protein in formic acid and by decreasing concentrations of DOPA and tyrosine in hydrolysates of periostracum (Fig. 2). DOPA and

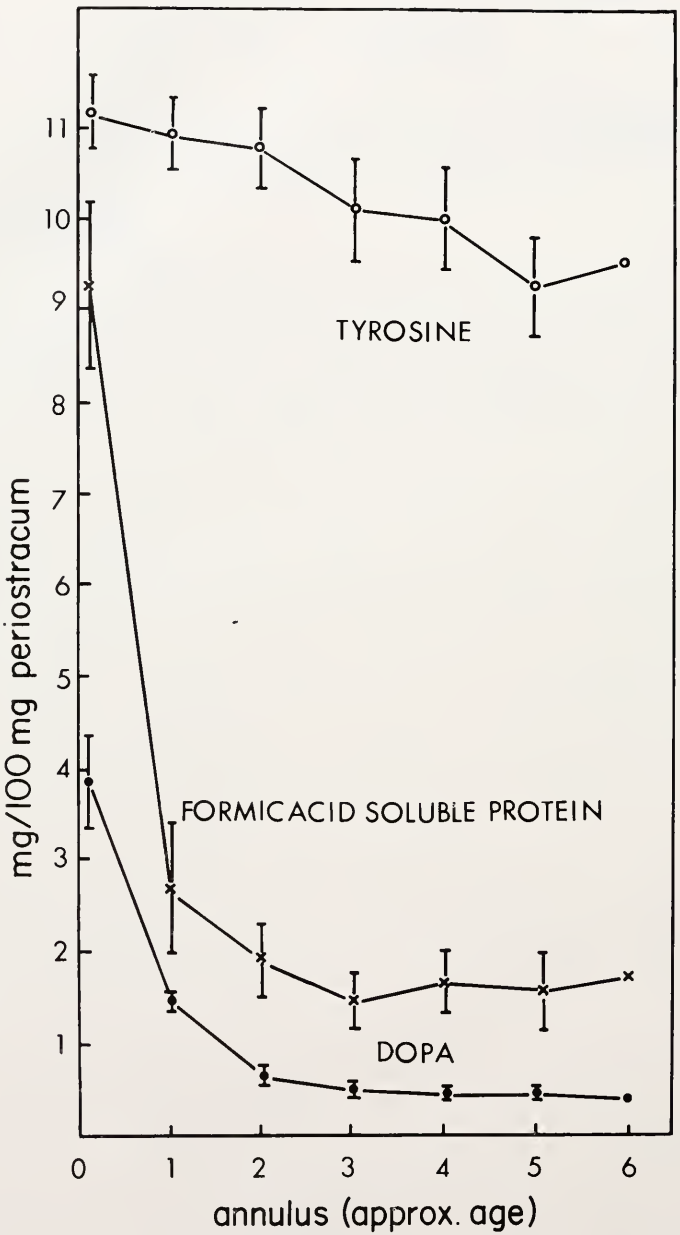

Figure 2. Variation of DOPA, tyrosine and protein solubility in periostracum with respect to the age of the annular section. Each point (excepting the last) represents the mean of 6 to 8 trials. Bars define the standard error of the mean. Numbers in the abscissa denote the *average age* of that annulus (years).

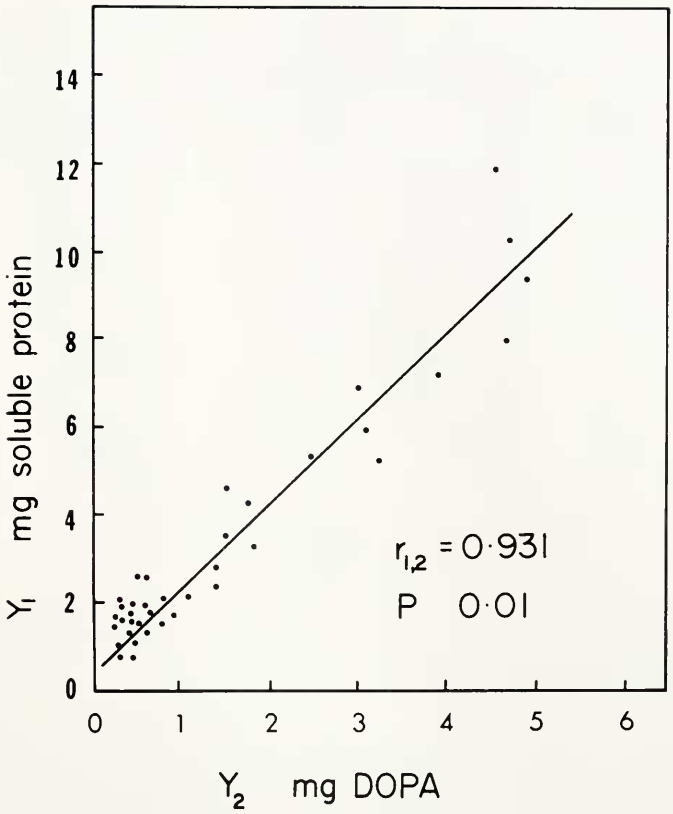

FIGURE 3. Plot of the correlation of DOPA and soluble protein in *Mytilus* periostracum. Dots represent 39 observations in which protein solubility (per 100 mg periostracum) in formic acid (Y_1) is plotted against its DOPA content (Y_2) determined after complete hydrolysis of the protein. The product-moment correlation coefficient is designated $r_{1,2}$ and was calculated according to Sokal and Rohlf (1969) from all the points in the distribution. P denotes probability that linear distribution is significant.

protein solubility exhibit decreases that resemble first order decay rates with asymptotes at 0.5 and 1.5% of weight, respectively. In contrast, tyrosine does not decrease significantly ($t_s = 1.323$, $df = 11$) between the margin and the first annulus; subsequently, its decrease is gradual and occurs throughout the life span of the periostracum.

Formic acid (13 M) is an effective protein denaturant and solvent, and for this reason, was chosen to extract all periostracal proteins not stabilized by covalent bonds. Figure 2 illustrates that, with the exception of the margin, very little protein can be extracted from periostracal annuli. The curves for protein solubility and DOPA in periostracum are sufficiently similar to suggest that a correlation exists between the two variables. Figure 3 shows a plot of all paired DOPA-protein solubility measurements. The data exhibit a roughly linear distribution ($R = 0.931$) and support the interpretation that a decrease in recoverable DOPA is directly proportional to decreasing protein solubility. The chief advantage of this correlation plot is that it eliminates the need for the most approximately measured variable, *i.e.*, age.

TABLE I

Amino acid composition of soluble, marginal and annular periostracum reported as percent weight of total weight.

	Soluble % (g/g)	Marginal % (g/g)	1st Annulus % (g/g)	1st Annulus / Margin
Asp	2.2	4.3	4.5	1.05
Thr*	1.4	1.2	1.0	0.83
Ser*	2.6	3.4	2.7	0.75
Glu	2.5	2.3	2.1	0.91
Pro	2.0	1.8	1.7	0.94
Cly	22.9	26.5	25.9	0.98
Ala	1.6	2.0	1.7	0.85
Cys/2	0.4	0.3	0.4	1.33
Val	2.5	3.3	3.3	1.00
Met	0.3	0.3	0.2	0.67
Ile	0.5	1.3	1.3	1.00
Leu	1.5	2.1	2.4	1.14
DOPA*	4.3	4.0	1.8	0.46
Tyr*	11.3	11.1	11.6	1.04
Phe	2.7	2.3	2.2	0.96
His	0.9	1.2	1.1	0.92
Lys	1.5	1.5	0.7	0.47
Arg	3.3	4.0	3.8	0.95
Total	64%	74%	68%	

* Corrected for losses due to hydrolysis.

Table I shows the per cent weight recovery of other amino acids from soluble, marginal and first annular periostracum and illustrates two significant points. The first is that formic acid-soluble protein from the periostracal margin is similar to the whole periostracum in amino acid composition. Both contain high concentrations of glycine and tyrosine; both contain DOPA. The other point in Table I is a comparison of the amino acid compositions of the margin and first annulus. In view of the drastic decrease in both DOPA and protein solubility between the margin and first annulus, we were particularly interested in determining what other, if any, amino acids decreased in the same interval. In agreement with Figure 2, results in Table I illustrate that tyrosine does not change significantly while DOPA is diminished by nearly 50%. Ignoring variation due to rounding off, most amino acids are as abundant in hydolysates of the first annulus as they are in the margin. Only lysine appears to be diminished by the same extent as DOPA, but the significance of this is difficult to assess because of the relatively low concentrations of lysine to begin with.

It is noteworthy that the weight recovery of periosracum after acid hydrolysis never approaches 100%. The balance of weight unaccounted for could consist of labile amino acids (tryptophan), sugars, pigments, residual water, lipids or mineral salts. A substantial yet still undefined portion of the periostracal hydrolysate appears to exist as ultraviolet light-absorbing aromatic material. In addition to the amino acids phenylalanine, tyrosine and DOPA, which are the first three peaks (A-C) to elute from a column of Bio-Gel P-2 (Fig. 4), there are at least seven other distinct peaks. D is known to be tryptophan, and H to be 6-bromotryptophan (Andersen, unpublished results). The apparent ability of some tryptophan to resist destruction during the acid hydrolysis of periostracum is not understood. Of the remaining peaks, E and G have been further char-

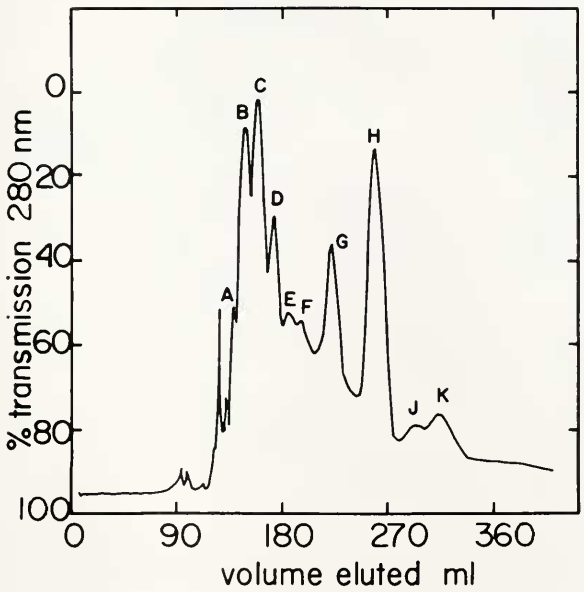

FIGURE 4. Elution of UV-absorbing compounds from acid hydrolysed marginal periostracum (50 mg) on a column of Bio-Gel P-2. Buffer is 0.2 M acetic acid and the flow rate is 12.5 ml/hr. Fractions containing peaks E and G were pooled for further purification by ion exchange on cellulose phosphate.

acterized and may shed some light on the fate of DOPA. E and G elute 2 and 6 hr, respectively after DOPA on cellulose phosphate using an ion gradient of 0.4 to 1.0 M NaCl according to Waite and Andersen (1978). On cellulose thin-layer chromatography, E and G migrate as uniform ninhydrin-positive spots with R_f values of 0.48 and 0.55, respectively, compared to 0.31 for DOPA. The ultraviolet spectra of the compounds between 225 to 325 nm, showed λ_{max} for both at 250 nm, and shoulders at 279 (E) and 284 nm (G). These spectra resemble strikingly some of the spectral characteristics of DOPA addition compounds with cysteine (Ito and Prota, 1977; Agrup, Hansson, Rorsman, Rosengren and Rosengren, 1976), and further research is expected to resolve whether these compounds might be DOPA-derived cross-links.

DISCUSSION

Insolubility, in addition to mechanical strength and resistance to proteolysis, is a key characteristic of scleroproteins (Linderstrøm-Lang and Duspiva, 1936). In the periostracum of *Mytilus edulis,* only newly secreted marginal material contains a substantial amount of protein soluble in formic acid; the remainder is almost wholly insoluble in the same solvent. Thus, it would appear that sclerotization occurs rapidly in *Mytilus.* Our results indicate that the degree of sclerotization of periostracal protein is closely correlated to the concentration of L-DOPA in acid hydrolysates of the protein.

Few will contest the premise that DOPA contributes to quinone-tanning. It is an immediate quinone precursor by enzymatic catalysis or auto-oxidation (Mason, 1948), and DOPA quinones can condense with amino acid residues to form

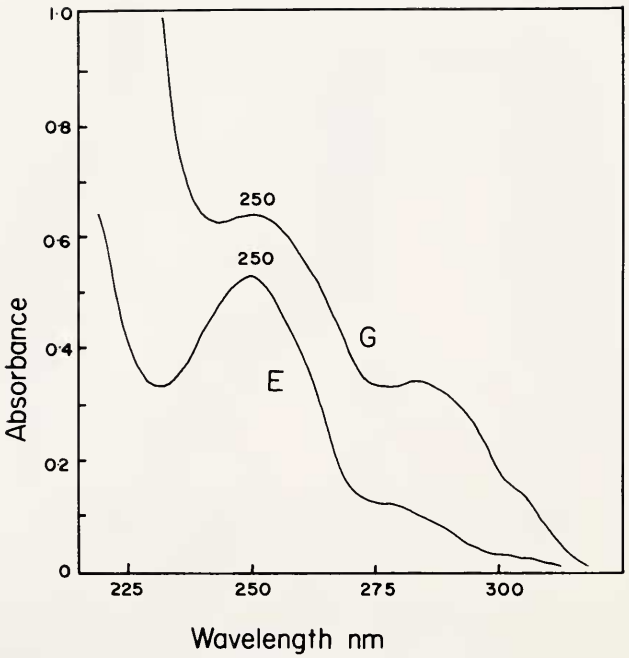

FIGURE 5. UV-spectra of purified peaks E and G. Maximal absorbance occurs at 250 nm. Solvent was 0.2 M acetic acid.

quinone adducts (Mason and Peterson, 1965; Ito and Prota, 1977). A problem invariably arises, however, with respect to whether tyrosine or DOPA actually serves as the precursor for quinones. Many of the current assumptions about sclerotization have been influenced by the studies of Lissitzky, Rolland, Reynaud and Lasry (1962) showing that DOPA transiently present in proteins *in vitro* is derived from tyrosyl residues by the direct action of mushroom polyphenoloxidase. Without compelling evidence, Pujol (1967) compared this reaction to the sclerotization of tyrosine-rich protein in *Mytilus* byssus. Similarly, many workers have since relied on the same reaction (often called autotanning) to explain sclerotization of tyrosine-rich proteins in molluscan periostracum.

We consider the mushroom polyphenoloxidase reaction as inadequate for describing sclerotization of periostracum. The following evidence now exists supporting a direct role of DOPA, not tyrosine, in the sclerotization of mytilid periostracum: it is known that periostracal phenoloxidase does *not* catalyze the oxidation of tyrosine (Waite and Wilbur, 1976); that catecholic compounds (DOPA) are abundant in the periostracum-secreting cells of the mantle (Bubel, 1973); that DOPA is present at proportions of up to 4% in a soluble presclerotin (MW 20,000) of formic acid-extracted periostracum (Waite, Saleuddin and Andersen, 1979), and that DOPA, not tyrosine, decreases during the major period of sclerotization between the margin and first annulus. Of course, DOPA still owes its existence to a post-translational modification of some tyrosyl residues by o-hydroxylation (Waite, Saleuddin and Andersen, 1979), but this event probably occurs during cellular processing of the protein, prior to its involvement in sclerotization.

Hunt and Oates (1978) have avoided the DOPA-tyrosine question by emphasiz-

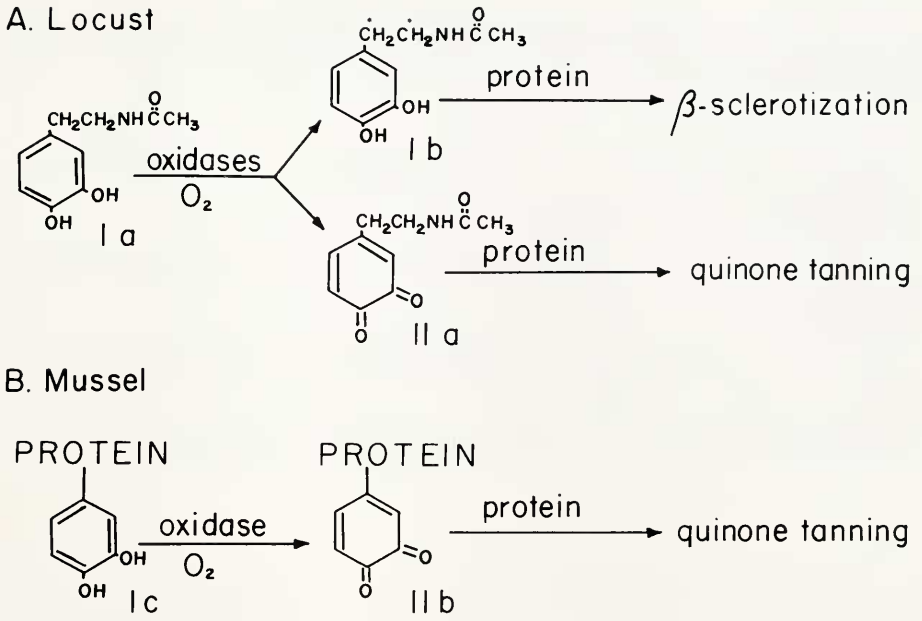

FIGURE 6. Sclerotization in locust cuticle (A) and *Mytilus* periostracum (B). Asterisks in A signify side chain carbons activated during β-sclerotization. Structures Ia-c are catechols: II a–b are quinones.

ing the products of aromatic cross-links in molluscan periostracum rather than the precursors. Unfortunately, nothing is known about the fate of DOPA following its oxidation to quinones in periostracum. One would assume that DOPA-quinones react with certain amino acid residues to form intermolecular cross-links. Lysine may be such a reactive residue, but our results are not yet conclusive about this. The observation by Morrison, Steele and Danner (1969) that quinones *in vitro* are capable of reacting with almost all amino acids could portend great difficulty in the isolation of specific cross-links from periostracum.

Sclerotization of exoskeletal proteins has been exploited throughout the animal kingdom, producing structures with a diversity of physical properties appropriate to the needs of the organism. A comparison of sclerotization of locust cuticle with that of *Mytilus* periostracum reveals striking similarities as well as differences. In both, the oxidation of catecholic precursors mediates protein sclerotization. However, in the locust cuticle (Fig. 6-A), the catechol, N-acetyldopamine, is of low molecular weight and freely diffusable. During sclerotization, it can be converted to either a quinone or a catechol with activated side chain (β-sclerotization). Thus, in the locust, sclerotization can be modulated by the local concentration of N-acetyldopamine relative to protein, and by the ratio of quinone-tanning to β-sclerotization (Andersen, 1974). In *Mytilus* periostracum (Fig. 6-B), protein with tightly bound DOPA residues is secreted from the mantle and cross-linked as the oxidized DOPA quinones react with available nucleophilic groups. Although less sophisticated than sclerotization in locust cuticle, the DOPA protein seems ideally suited to sclerotization of *Mytilus* periostracum; it is water-insoluble, reactive as a monomer, inert as polymer, frugal (catechol and protein in one) and can be secreted continuously without the need for exuviation.

Thanks are due to Mr. Tim Housley for his assistance in analyzing the amino acid composition and to the National Science Foundation (Grant SMI 76–17904) for financial support.

Summary

1. The periostracum of *Mytilus edulis* is a highly sclerotized protein structure as demonstrated by its low solubility in 13 M formic acid.

2. The concentration of DOPA in periostracal hydrolysates is not uniformly distributed, but varies directly with protein solubility and logarithmically with the approximate age of the material.

3. The results suggest that the disappearance of DOPA is linked to the sclerotization of periostracum, probably by way of quinone-tanning; tyrosine does not appear to participate in sclerotization.

4. Sclerotization of DOPA-protein in *Mytilus* periostracum is compared to a similar process in the exoskeleton of the locust, *Schistocerca gregaria.*

LITERATURE CITED

Agrup, G., C. Hansson, H. Rorsman, A. M. Rosengren, and E. Rosengren, 1976. Mass spectrometric analysis of enzymatically synthesized cysteinyldopa isomers. *Communications of the Department of Anatomy*, Unversity of Lund, Sweden, **5**: 1–17.

Andersen, S. O., 1974. Evidence for two mechanisms of sclerotization in insect cuticle. *Nature*, **251**: 507–508.

Brown, C. H., 1952. Some structural proteins of *Mytilus edulis* L. *Q. J. Micros. Sci.*, **93**: 487–502.

Brown, C. H., 1950. Quinone-tanning in the animal kingdom. *Nature*, **165**: 275.

Bubel, A., 1973. An EM investigation into the distribution of polyphenols in the periostracum and cells of the inner face of the outer fold of *Mytilus edulis*. *Mar. Biol.*, **23**: 2–15.

Degens, E. T., and D. W. Spencer, 1966. Data file on amino acid distribution in calcified and uncalcified tissues of shell-forming organisms. *Technical Report No.* 66–27, Woods Hole Oceanographic Institution.

Degens, E. T., D. W. Spencer, and R. H. Parker, 1967. The paleochemistry of molluscan shell proteins. *Comp. Biochem. Physiol.*, **20**: 553–579.

Hartree, E. F., 1972. Determination of protein. *Anal. Biochem.*, **48**: 422–428.

Hunt, S., and K. Oates, 1978. Fine structure and molecular organization of the periostracum in a gastropod mollusc *Buccinum undatum* L. and its relation to similar structural protein systems in other invertebrates. *Phil. Trans. R. Soc. Lond., Biol. Sci.*, **283B**: 417–463.

Ito, S., and G. Prota, 1977. A facile one-step synthesis of cysteinyldopas using mushroom tyrosinase. *Experientia*, **33**: 1118–1199.

Knight, D .P., and S. Hunt, 1974. Molecular and ultrastructural characteristics of the egg capsule of the leech *Erpobdella octoculata* L. *Comp. Biochem. Physiol.*, **47A**: 871–880.

Linderstrøm-Lang, K., and F. Duspiva, 1936. Studies in enzymatic histochemistry. XVI The digestion of keratin by larvae of the clothes moth (*Tinola bisselliela* Humm.). *C. R. Trav. Lab. Carlsberg, Ser. Chim.*, **21**: 1–82.

Lissitzky, S., M. Rolland, J. Reynaud, and S. Lasry, 1962. Oxydation de la tyrosine et de peptides ou protéines la contenant par la polyphenoloxydase de champignon. III. Propriétés des protéines oxydées et de certaines DOPA-protéines. *Biochim. Biophys. Acta*, **65**: 481–494.

Lutz, R. A., 1976. Annual growth patterns in the inner shell of *Mytilus edulis* L. *J. Mar. Biol. Assoc. UK.*, **56**: 723–731.

Mason, H. S., 1948. Chemistry of melanin: III. Mechanism of oxidation of dihydroxyphenylalanine by tyrosinase. *J. Biol. Chem.*, **172**: 83–99.

Mason, H. S., and E. W. Peterson, 1965. Reactions between enzyme-generated quinones and amino acids. *Biochim. Biophys. Acta*, **111**: 134–146.

Moore, S., 1968. Amino acid analysis: aqueous dimethylsulfoxide as solvent for the ninhydrin reaction. *J. Biol. Chem.*, **243**: 6281–6283.

Morrison, M., W. Steele, and D. J. Danner, 1969. The reaction of benzoquinone with amines and proteins. *Arch. Biochem. Biophys.*, **134**: 515–523.

Nagatsu, T., 1973. *Biochemistry of Catecholamines.* University Park Press, Tokyo, 362 pp.

Pujol, J. P., 1967. Le complexe byssogéne des mollusques bivalves. Histochimie comparée des secrétions chez *Mytilus edulis* L. et *Pinna nobilis* L. *Bull. Soc. Linn. Normandie*, **8**: 308–332.

Rainsford, K. D., 1967. As cited in Clegg, J. A., and J. D. Smyth, 1968. Platyhelminth growth and culture, in *Chemical Zoology*. Vol. V, Academic Press, New York, p. 435.

Sokal, R. R., and F. J. Rohlf, 1969. *Biometry*. W. H. Freeman Co., San Francisco, 494 pages.

Waite, J. H., 1977. Evidence for the mode of sclerotization in a molluscan periostracum. *Comp. Biochem. Physiol.*, **56B**: 157–162.

Waite, J. H., and S. O. Andersen, 1978. 3,4-Dihydroxyphenylalanine in an insoluble shell protein of *Mytilus edulis*. *Biochem. Biophys. Acta*, **541**: 107–114.

Waite, J. H., and K. M. Wilbur, 1976. Phenoloxidase in the periostracum of the marine bivalve *Modiolus demissus* Dillwyn. *J. Exp. Zool.*, **195**: 339–368.

Waite, J. H., A. S. M. Saleuddin, and S. O. Andersen, 1979. Periostracin—A soluble precursor of sclerotized periostracum in *Mytilus edulis*. *J. Comp. Physiol.*, **130**: 301–307.